

Expression of Recombinant VP2 Protein of Canine Parvovirus in *Escherichia coli*

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ABSTRACT

Canine parvovirus (CPV) appears to be endemic in almost all populations of wild and domesticated dogs. It causes serious contagious enteric disease. The VP2 protein of CPV is a major capsid protein and plays an important role in the host immune response. In the present study, the recombinant VP2 was expressed in *Escherichia coli* (*E. coli*) using the pBAD expression system. Virus DNA from the infected feces was extracted and used to amplify the whole VP2 gene by using specific primers. Subsequently, the whole VP2 gene was ligated with plasmid pBAD202/D-TOPO and used to transform into the *E. coli* strain TOP10. The SDS-PAGE analysis revealed a specific band approximately 80 kDa and was found mainly in the pellet of the bacterial lysate. The optimum time and concentration of arabinose for expression of recombinant VP2 protein was 8 h and 0.002%, respectively. By dot blot and Western blot analysis, the recombinant VP2 protein showed specific interaction with mouse anti-histidine monoclonal antibody and rabbit anti-CPV hyperimmune serum. The recombinant protein VP2 might be a useful tool for the development of a diagnostic test for the detection of CPV and a vaccine against CPV.

Keywords: canine parvovirus, VP2 protein, *Escherichia coli*

INTRODUCTION

Canine parvovirus type 2 (CPV-2) was first identified in the USA in 1978 as the cause of hemorrhagic enteritis and myocarditis in dogs and it has been known as an enteric pathogen of dogs throughout the world (Appel *et al.*, 1979). During 1979 to 1981, an antigenic variant CPV-2a was found and a second variant CPV-2b was demonstrated in 1984. However, both variants can infect, replicate and transmit between dogs and cats as CPV-2 (Parrish *et al.*, 1991). Recently,

CPV-2c has been identified in parts of Europe, America and Asia (Ikeda *et al.*, 1999; Buonavoglia *et al.*, 2001; Kapil *et al.*, 2007; Perez *et al.*, 2007).

CPV is a small and non-enveloped virus containing approximately 5.2 kb of a single-stranded DNA (Parrish *et al.*, 1991). CPV can be divided into different biotypes of 1, 2, 2a, 2b and 2C, based on the differentiation of some amino acid residues of the virus capsid protein. The genome encodes two nonstructural proteins (NS1 and NS2) and three structural proteins (VP1, VP2 and VP3) that are called capsid proteins. CPV

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capsid proteins are composed of VP1 (82 kDa), VP2 (65 kDa) and VP3 (the proteolytic processing of the N-terminal of VP2; 63 kDa) (Paradiso *et al.*, 1982; Reed *et al.*, 1988). VP2 is a major capsid protein and plays an important role in the determination of antigenicity and the host range of CPVs. Epitope mapping experiments showed that all epitopes generating neutralizing antibody are within VP2 (Turiso *et al.*, 1991). The current study focused on the expression of recombinant VP2 protein and the characterization of recombinant VP2 protein by Western blot analysis, using mouse anti-histidine monoclonal antibody and rabbit hyperimmune serum against CPV.

MATERIALS AND METHODS

Virus DNA

Virus DNA was obtained from fecal samples of dogs that had symptoms of nausea and hemorrhagic diarrhea. The fecal samples were used for DNA extraction using the phenol-chloroform extraction method described by Sambrook and Russell (2001). Briefly, 100 μ L of fecal sample was resuspended in deionized water, mixed with 500 μ L of D-solution (4 M guanidium thiocyanate, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0) and incubated at room temperature for 5 min. Subsequently, 200 μ L each of phenol and chloroform were added, vortexed and centrifuged at 13,000 rpm for 5 min. Total DNA was precipitated with 700 μ L of absolute ethanol and was washed with 75% ethanol. Finally, the DNA pellet was air-dried and resuspended in 30 μ L of TE buffer (pH 8.0).

Amplification of VP2 gene

Specific primers for the amplification of the full-length VP2 gene of CPV were designed based on VP2 sequences from the GenBank database. The forward primer (F-VP2) sequence was 5' CACC ATG AGT GAT GGA GCA GTT CAA C 3' and the reverse primer (R-VP2) was 5'

ATA TAA TTT TCT AGG TGC TAG 3'. The expected size of PCR products was 1,755 bp. The PCR mixture was composed of 1x Pfx amplification buffer (5 mM Tris-HCl (pH 8.0), 5 mM KCl₂, 0.1 mM DTT, 0.01 mM EDTA and 5% (v/v) glycerol), 0.3 mM dNTPs, 1 mM MgSO₄, 100 pmol of each forward and reverse primer, 2.5 U *Taq* DNA polymerase (InvitrogenTM) and 10 μ L of DNA template to give a total volume of 100 μ L. For the negative control, 10 μ L of distilled water was substituted for the DNA template amount to give a total volume of 100 mL. The PCR condition was pre-denatured at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, elongation at 72°C for 60 s and a final extension at 72°C for another 10 min.

Cloning of VP2 gene into plasmid pBAD202/D-TOPO[®]

The PCR products were purified using a QIAquick[®] Gel Extraction Kit (Qiagen[®]) and the purified PCR products were subsequently ligated with pBAD202/D-TOPO[®] (Invitrogen[®]) according to the manufacturer's protocol. Briefly, purified PCR products were mixed with 1 μ L of salt solution (1.2 M NaCl₂, 60 mM MgCl₂), 4 μ L of pBAD202/D-TOPO[®] and 1mL of distilled water. The ligation mixture was gently mixed and incubated at room temperature for 15 min and then transformed into One shot[®]TOP10 competent cells (Invitrogen[®]). Subsequently, 250 mL of SOC medium was added to the transformed *E. coli* and shaken at 200 rpm for 1 h before spreading on LB agar containing 50 mg/mL of kanamycin. The recombinant plasmids were determined by PCR and sequencing.

Protein expression

The inoculums were prepared by adding 100 μ L of recombinant *E. coli* stock in 2 mL LB broth containing 50 mg/mL kanamycin and shaken at 200 rpm at 37°C overnight. Subsequently, 150

μ L of inoculum was added to each of five tubes containing 10 mL LB broth with 50 mg/mL kanamycin. The tubes were shaken for another 4-5 h until the optical density (OD_{600}) reached a value of approximately 0.5. Then, the optimum concentration of arabinose for recombinant VP2 expression was determined by adding 2, 0.2, 0.02, 0.002 and 0.0002% of arabinose into samples of culture medium. The cultures were then sampled every 2 h and kept at 80°C for further verification by SDS-PAGE. Control samples were also made using the same conditions but without the addition of arabinose. The recombinant VP2 protein was also partially purified using Ni-NTA beads, according to the manufacturer's recommendation (Qiagen®).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

One microliter of induced recombinant *E. coli* pellet was added to 5 μ L of SDS sample buffer (250 mM Tris HCl, 20% glycerol, 4% SDS, and 0.006% bromophenol blue), boiled for 5 min and centrifuged at 5,000 rpm for 5 min. The supernatants were loaded into 10% SDS-PAGE gel and electrophoresed for 90 min at 110 volts. The gel was then stained with staining solution (0.01 % Coomassie Brilliant Blue, 50% methanol, 40% distilled water, and 10% acetic acid) for 10 min and subsequently de-stained with de-staining solution (50% methanol, 40% distilled water, and 10% acetic acid).

Preparation of polyclonal antibody against CPV

The polyclonal antibody against CPV was prepared by immunizing three rabbits with 1 mL of Canine Parvovirus modified-lived vaccine (Quantum® Dog Pv, Schering-Ploug) at week 0, 2, 4, and 6. The rabbits were bled at week 8 for post immunization. The sera were collected by low-speed centrifugation. The rabbit hyperimmune serum against CPV was used for detection of

reactivity against CPV by immunoblot.

Western blot analysis

The recombinant VP2 protein was separated by 10% SDS-PAGE gel and electro-transferred onto nitrocellulose membrane at 400 mAmp for 300 min. The membrane was blocked with 5% skim milk at 4°C overnight. The membranes were then incubated with either 1:500 mouse anti-histidine monoclonal antibody (Sigma®) or 1:100 rabbit anti-CPV hyperimmune serum for 1 h at room temperature. Subsequently, the membranes were washed and incubated with either 1:200 goat anti-mouse IgG conjugated with horseradish peroxidase (KPL) or 1:300 goat anti-rabbit IgG conjugated peroxidase (Sigma®) for 1 h at 37°C. After washing, the membranes were incubated with 3 mL of 0.6 mg/ml diaminobenzidine (DAB, Sigma®) containing 0.03% H_2O_2 for 5-10 min at room temperature. The recombinant VP2 protein was visualized as a brown band on nitrocellulose membrane.

RESULTS AND DISCUSSION

The VP2 gene of CPV was successfully amplified. The size of PCR products was approximately 1,700 bp (Figure 1). The electrophoresis technique showed only an

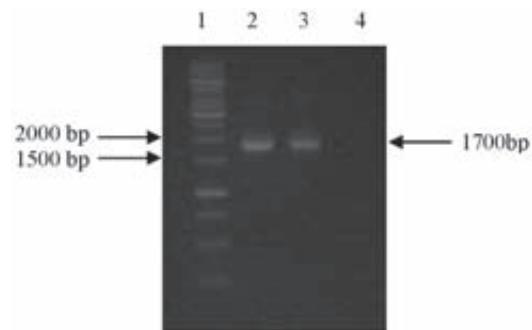


Figure 1 1% Agarose gel electrophoresis of the PCR products of VP2 gene. Lane 1 = kb DNA marker; lanes 2 and 3 = VP2 gene; and lane 4 = negative control.

approximate range and did not express a gene orientation. Therefore, the cloned VP2 gene was sequenced that resulted in a size of 1,755 bp with the correct orientation. The PCR products were cloned into pBAD202/D-TOPO® and the positive clones showed PCR products about 1,900 bp in length (Figure 2). The presence of the VP2 gene in the recombinant plasmid was confirmed by sequencing (accession no. FJ869126).

Several factors are relevant in the selection of the expression system and they depend largely on the biochemical and biological properties of the protein of interest, the amount of recombinant protein required and on cost-efficient production systems (Geisse *et al.*, 1996). In previous studies, recombinant VP2 protein has been successfully expressed in many systems, such as in an insect cell (SF9) for baculovirus expression system (Saliki *et al.*, 1992; Gilbert *et al.*, 2005) and the 10 µg protein was utilized as a vaccine in dogs (Turiso *et al.*, 1992; Moonjiti, 2006). The recombinant VP2 expressed in *E.coli* was applied to dogs by injecting as 100 µg DNA with and without adjuvant. The dogs injected with recombinant plasmid were protected fully from the disease (Gupta *et al.*, 2005). The expressed proteins had the capability to induce protective immunity. Therefore, *E. coli* is an interesting system to express recombinant VP2 due to the low

cost of expression (Park *et al.*, 2007; Zeng *et al.*, 2008).

In the current study, the *E. coli* expression system was selected because it was cheap, fast and easy to manipulate. The plasmid pBAD202/D-TOPO provides high ligation efficiency due to the attached topoisomerase at both ends of this linear plasmid (Invitrogen, 2004). The directional ligation of a blunt-end PCR product saves time in confirming gene orientation, so only a PCR technique was needed to determine the inserted gene. Then, the selected colonies were ready for protein expression.

According to the pBAD202/D-TOPO® expression system, the concentration of arabinose and the induction time can influence the amount of expression of recombinant protein. In this experiment, the optimum concentration of arabinose and the optimum induction time were 0.002% (Figure 3) and 8 h (Figure 4), respectively. The molecular weight of the VP2 protein was calculated by summation of its amino acids (Adams, 2003), which equaled 65 kDa. The calculated molecular weight of recombinant VP2 protein, expressed using recombinant *E. coli*, was 80 kDa, due to the extra molecular weight of thioredoxin and 6xhistidine included in the plasmid pBAD 202/D-TOPO. The recombinant VP2 protein was found mostly in the insoluble

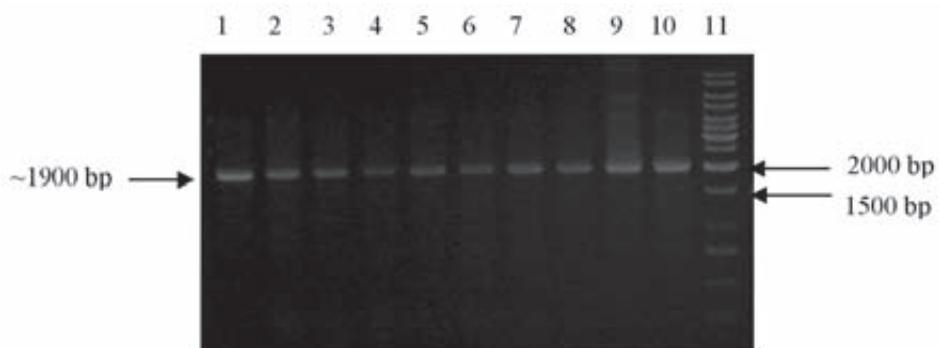


Figure 2 1% Agarose gel electrophoresis of the PCR products of the recombinant VP2 plasmids. Lanes 1 to 10 = PCR products from the positive clones; and lane 11 = 1 kb DNA marker.

fraction that had been identified in a previous report (Park *et al.*, 2007). The molecular weight of recombinant VP2 protein was approximately 80 kDa on 10% polyacrylamide (Figures 3 and 4), similar to the purified recombinant VP2 protein (Figure 5).

By dot blot analysis, the crude protein showed interaction with both mouse anti-histidine

IgG monoclonal antibody and rabbit anti-CPV polyclonal antibody (Figures 6A and 7A). For Western blot analysis, using mouse anti-histidine IgG monoclonal antibody and rabbit anti-CPV polyclonal antibody, the crude protein also showed a positive band at approximately 80 kDa (Figures 6B and 7B). The purified VP2 protein was also reacted specifically with both antibodies (Figures

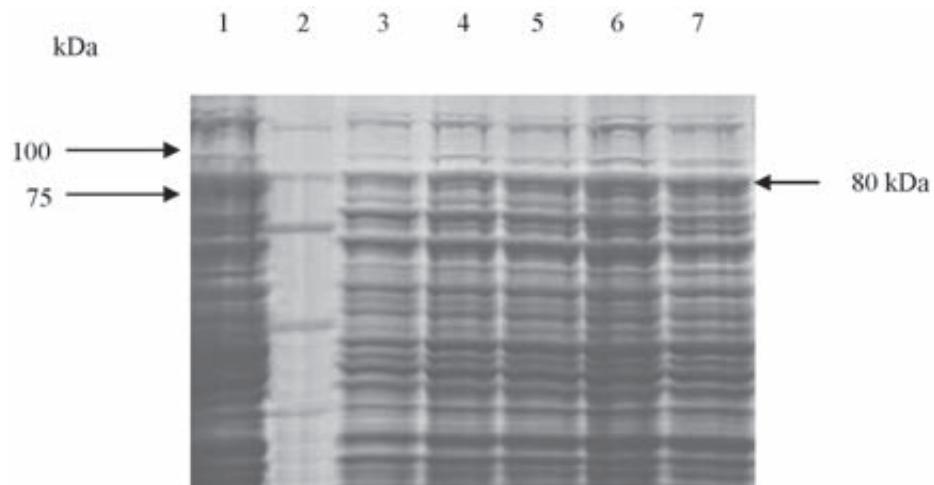


Figure 3 10% SDS-PAGE analysis of the optimum concentration of arabinose for the induction of recombinant VP2 protein. Lane 1 = wild type protein; lane 2 = the molecular protein marker; and lanes 3-7 = 2, 0.2, 0.02, 0.002 and 0.0002% arabinose, respectively.

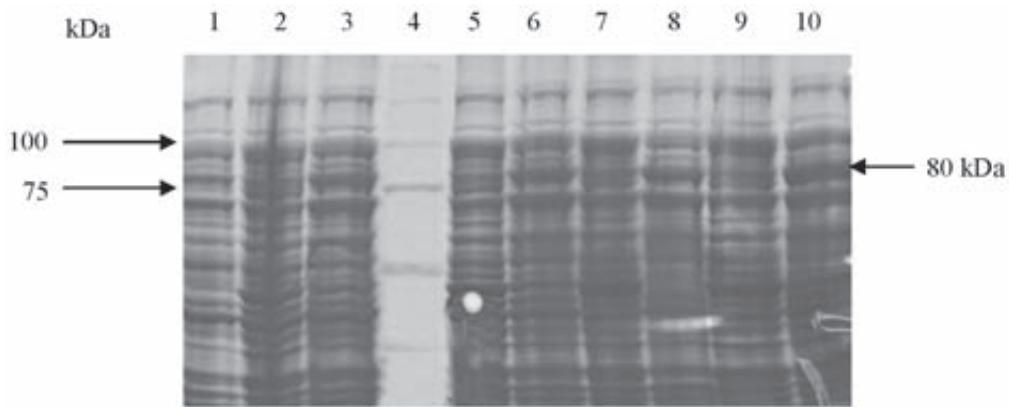


Figure 4 10% SDS-PAGE analysis of the optimum time for the induction of recombinant VP2 protein. Lanes 2, 5, 7 and 9 = wild type protein at 2, 4, 6, 8 h; lanes 1, 3, 6, 8, 10 = recombinant VP2 protein at 0, 2, 4, 6, 8 h; and lane 4, the molecular protein marker.

8A and 8B). When the total and purified recombinant VP2 protein was detected with the rabbit anti-CPV hyperimmune serum, other bands lower than 80 kDa were found (Figures 7B and 8B), as was shown previously in Park *et al.* (2007). These bands may be generated either by proteolytic cleavage or degradation by host

protease, which has been described in previous reports (Paradiso *et al.*, 1982, 1984; Tullis *et al.*, 1992) or the VP2 may contain sequences that cause premature degradation of the gene products in *E. coli* (Park *et al.*, 2007). Immunoblots of these membranes using rabbit anti-CPV hyperimmune serum showed more than one band. However, it proved that the recombinant VP2 protein had epitopes that reacted specifically with rabbit anti-CPV hyperimmune serum.

In conclusion, the VP2 gene of CPV was cloned into plasmid pBAD202/D-TOPO. A recombinant VP2 protein was successfully expressed in the *E. coli* expression system. The optimum concentration of arabinose and the induction period for the expression of recombinant VP2 protein was 0.002% and 8 h, respectively. The recombinant VP2 protein at the 80 kDa band interacted specifically with rabbit anti-CPV polyclonal antibodies and mouse anti-histidine IgG monoclonal antibody. Based on these results, the study established a procedure to produce immunogenic VP2 protein of CPV using a prokaryotic expression system. The recombinant VP2 protein in this study had specific interaction

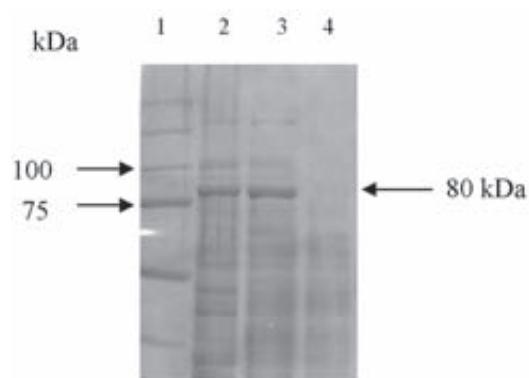


Figure 5 10% SDS-PAGE analysis of the purified recombinant VP2 protein. Lane 1 = the molecular protein marker; lane 2 = crude rVP2 protein; lane 3 = purified rVP2 protein; and lane 4 = purified wild-type protein.

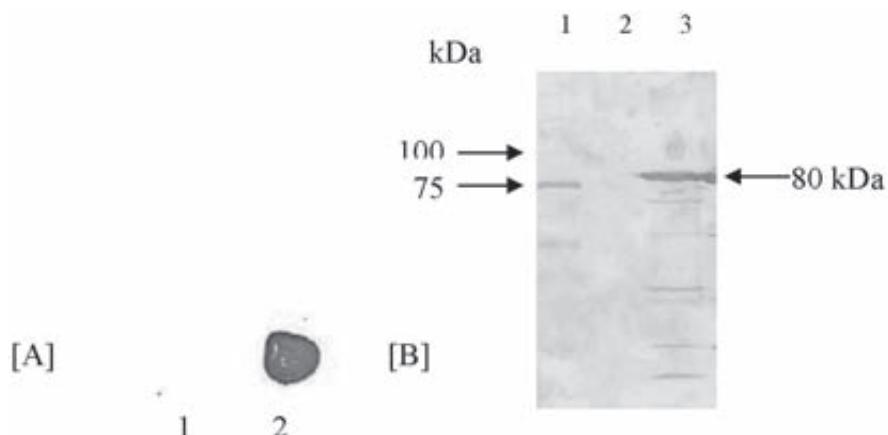


Figure 6 Dot blot (A) and Western blot (B) analysis of recombinant VP2 protein using mouse anti-histidine monoclonal antibody. By dot blot analysis, the recombinant VP2 protein was positive (lane 2) and the negative control was negative (lane 1). By Western blot analysis, lane 1 was the molecular protein marker, the negative control was negative (lane 2) and the recombinant VP2 protein showed positive results (lane 3).

with anti-CPV antibodies. Therefore, this recombinant protein could be considered as an interesting alternative for further detection of CPV infection and potential production of a subunit vaccine against CPV.

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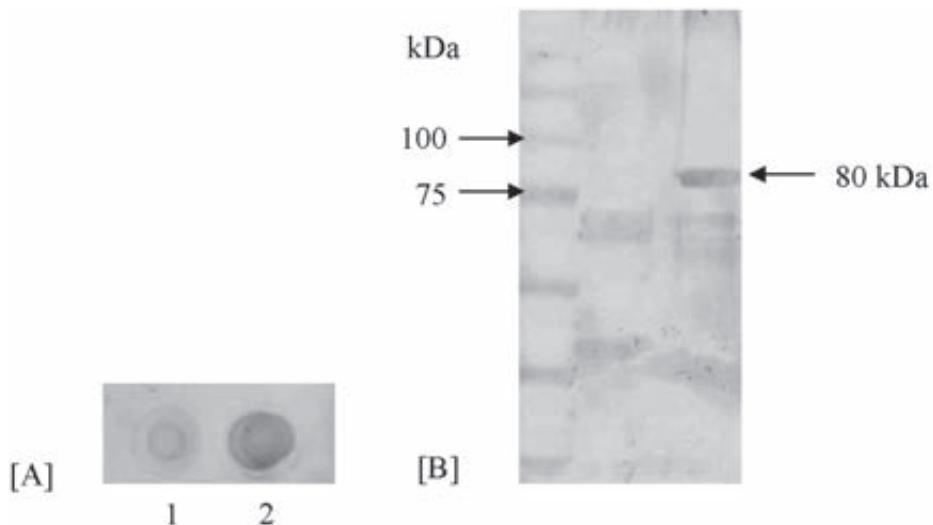


Figure 7 Dot blot (A) and Western blot (B) analyses of recombinant VP2 protein using rabbit anti-CPV polyclonal antibodies. (A) lane 2 = the recombinant VP2 protein; lane 1 = the negative control. (B) lane 1 = molecular protein marker; lane 2 = the negative control; and lane 3 = the recombinant VP2 protein.

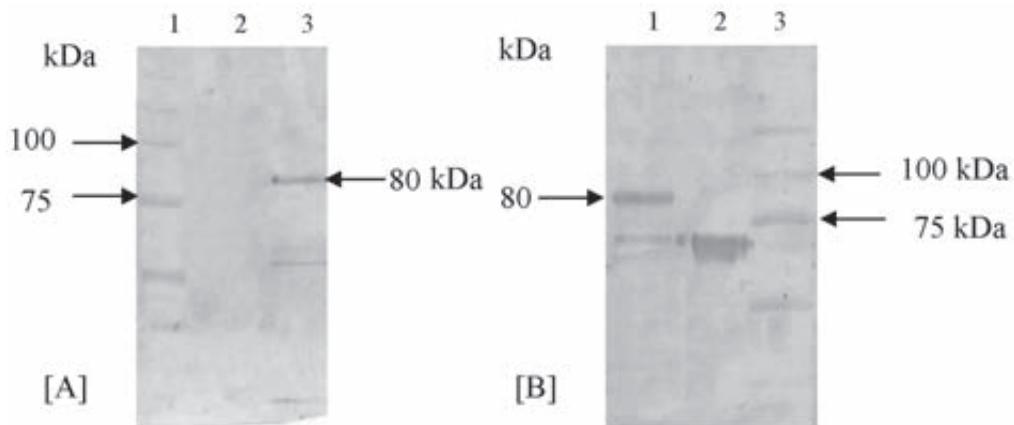


Figure 8 Dot blot and Western blot analysis of purified recombinant VP2 protein using mouse anti-histidine monoclonal antibody (A) and rabbit anti-CPV polyclonal antibodies (B). (A) lane 1 = molecular prestain protein marker; lane 2 = wild type protein; and lane 3 = purified rVP2 protein. (B) lane 1 = purified rVP2 protein; lane 2 = wild type protein; lane 3 = molecular prestain protein marker.

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